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16. ABSTRACT

The biodegradation of o-, m-, and p-cresol was examined in material obtained from a shallow anaerobic alluvial sand aquifer. The cresol isomers were preferentially metabolized, with p-cresol being the most easily degraded. m-Cresol was more persistent than the para-isomer, and o-cresol persisted for over 90 days. Biodegradation of cresol isomers was favored under sulfate-reducing conditions (SRC) compared with that under methanogenic conditions (MC). Slurries that were acclimated to p-cresol metabolism transformed this substrate at 18 and 330 nmol/h per g (dry weight) for MC and SRC, respectively. Inhibition of electron flow to sulfate reduction with 2.0 mM molybdate reduced p-cresol metabolism in incubations containing sulfate. When methanogenesis was blocked with 5 mM bromoethanesulfonic acid in incubations lacking sulfate, p-cresol catabolism was retarded. Under SRC 3.4 mol of sulfate was consumed per mol of p-cresol metabolized. The addition of sulfate to methanogenic incubations stimulated p-cresol degradation. Simultaneous adaptation studies in combination with spectrophotometric and chromatographic analysis of metabolites indicated that p-cresol was oxidized under SRC to p-hydroxybenzoate via the corresponding alcohol and aldehyde. This series of reactions was inhibited under sulfate-limited or aerobic conditions. Therefore, the primary catabolic event for p-cresol decomposition under SRC appears to involve the hydroxylation of the aryl methyl group.

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# Biodegradation of Cresol Isomers in Anoxic Aquifers

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**The biodegradation of *o*-, *m*-, and *p*-cresol was examined in material obtained from a shallow anaerobic alluvial sand aquifer. The cresol isomers were preferentially metabolized, with *p*-cresol being the most easily degraded. *m*-Cresol was more persistent than the *para*-isomer, and *o*-cresol persisted for over 90 days. Biodegradation of cresol isomers was favored under sulfate-reducing conditions (SRC) compared with that under methanogenic conditions (MC). Slurries that were acclimated to *p*-cresol metabolism transformed this substrate at 18 and 330 nmol/h per g (dry weight) for MC and SRC, respectively. Inhibition of electron flow to sulfate reduction with 2.0 mM molybdate reduced *p*-cresol metabolism in incubations containing sulfate. When methanogenesis was blocked with 5 mM bromoethanesulfonic acid in incubations lacking sulfate, *p*-cresol catabolism was retarded. Under SRC 3.4 mol of sulfate was consumed per mol of *p*-cresol metabolized. The addition of sulfate to methanogenic incubations stimulated *p*-cresol degradation. Simultaneous adaptation studies in combination with spectrophotometric and chromatographic analysis of metabolites indicated that *p*-cresol was oxidized under SRC to *p*-hydroxybenzoate via the corresponding alcohol and aldehyde. This series of reactions was inhibited under sulfate-limited or aerobic conditions. Therefore, the primary catabolic event for *p*-cresol decomposition under SRC appears to involve the hydroxylation of the aryl methyl group.**

There is little doubt that groundwater resources are contaminated by the waste disposal and land-use practices of modern man (23). To assess accurately the risk associated with such activities, information is needed on the physiochemical and biological processes governing pollutant transport and fate in groundwater. While physiochemical processes may result in the partial breakdown of contaminating substances, microbial degradation is the only process that can result in the complete mineralization of these compounds.

Information has steadily emerged on the biodegradation abilities of aerobic microorganisms. However, the subsurface is often devoid of dissolved oxygen, particularly when shallow aquifers are contaminated with degradable organic matter. Relatively little is known about anaerobic bioconversions of organic compounds other than standard metabolites. This lack of metabolic information makes it difficult to predict the anaerobic fate of organic chemicals that enter subsurface environments.

Alkyl phenols are important examples of groundwater contaminants that are produced from a variety of sources. Phenol, *m*- and *p*-cresol, as well as *m*- and *p*-ethylphenol were previously found to be transported in an anaerobic leachate from a municipal landfill (25). Because the microbial metabolism of these compounds was apparently slower than their rate of migration, the investigators suggested that anaerobic conditions may facilitate the movement of phenolic compounds in groundwater. Creosote is another source of alkyl phenols and polynuclear aromatic compounds that are known to pollute potable aquifers (6, 7). The disappearance of the creosote phenolic constituents under both methanogenic (MC) and nitrate-reducing conditions could not be accounted for by dilution or adsorption alone (6). Biodegradation of the contaminating chemical structures was suggested as a possible mechanism of creosote dissolution (5–7). Similarly, biological activity was used to help explain the reduction in phenols, cresols, and xylenols from an anoxic aquifer contaminated by underground coal gasification activities (13). Although the removal of phenolic materials from anoxic aquifers has been attributed to biodegradation, little information is known on the possible metabolic pathways involved in such transformations.

Therefore, this study was designed to determine the type of anaerobic metabolism that simple alkyl phenols undergo in samples obtained from a shallow aquifer contaminated by leachate from a municipal landfill. The previously characterized aquifer contains two sites that differ with respect to the dominant processes governing carbon and electron flow (R. E. Beeman and J. M. Suflita, Microb. Ecol. in press). One site was actively methanogenic, whereas sulfate reduction predominated at the other. Therefore, the anaerobic decomposition of cresol isomers was studied under these conditions.

Results of this study may eventually help in predicting the transport and fate of cresols, as well as other alkyl aromatic compounds, that pollute anaerobic environments such as the subsurface. It may also help lead to strategies designed to biologically remediate aquifers contaminated with such materials.

## MATERIALS AND METHODS

**Study sites and sample collection.** The aquifer material used in this study was collected from two areas adjacent to the municipal landfill in Norman, Okla. The landfill is located above a shallow alluvial sand aquifer which receives leachate from the decomposing refuse. The two sampling sites have been described previously (30, 31; Beeman and Suflita, in press). The methanogenic site had low to undetectable levels of sulfate and elevated rates of methanogenesis relative to the sulfate-reducing site, which had sulfate levels ranging from 2 to 11 mM and an endogenous rate of methanogenesis several orders of magnitude slower than that of the methanogenic site.

Aquifer solids and groundwater were obtained by digging (1 to 1.8 m) to the top of the water table and hand-filling sterile mason jars to capacity. The jars were tightly sealed and transported to an anaerobic glove box within 5 h. For

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most experiments, aseptically obtained cores of aquifer solids were taken by inserting sterile plastic core barrels (10-cm inner diameter by 30 cm; polyvinyl chloride pipe) into the top of the water table. The cores were excavated from the aquifer, transported to the laboratory within 0.5 h, and extruded from the core barrels in an anaerobic glove box. The outer 2 cm of core material was then shaved with a sterile spatula to remove the potentially contaminated portions of the core. This procedure allowed us to obtain core material without contamination by surface microbes and minimized the exposure of cores to oxygen.

Large volumes of groundwater were also obtained by using a portable well. The well was made from a steel pipe (5-cm outer diameter by 1.5 m) which had holes (0.3 cm) drilled around its lower half. The bottom of the pipe was fitted with a conical steel cap, which allowed us to hammer the pipe into the aquifer. The inner wall of the well was lined with a fine mesh (0.15 cm) aluminum screen to prevent sand from plugging the flexible well tubing (1-cm outer diameter) inserted to the bottom of the pipe. The entire well was sterilized before it was inserted into the water table. In the field the tubing was connected to an anaerobic 20-liter carboy, which was preflushed with $O_2$-free nitrogen and sealed to maintain anaerobic conditions. The groundwater was pumped into the carboy with a peristaltic field pump. In this manner, large volumes of groundwater were obtained without apparent oxygen exposure or contamination by surface microorganisms.

**Incubation of cresol isomers.** Initial studies on the degradation of cresol isomers employed the nonaseptically obtained aquifer samples. Sterile serum bottles (160-ml capacity) were filled inside an anaerobic glove box with 50 ± 0.5 g (wet weight) of aquifer solids and 50 ml of groundwater from the respective sites. The groundwater was previously boiled for 3 min under an $O_2$-free atmosphere of 80% $N_2$–20% $CO_2$ to remove traces of oxygen. After cooling, the groundwater was amended with 1.0 mM $Na_2S \cdot 9H_2O$ as a reductant and 0.0001% resazurin as a redox indicator. After sealing the serum bottles with a 1-cm-thick rubber septa, the bottles were removed from the glove box, and the headspace gas was replaced with an 80% $N_2$–20% $CO_2$ mixture. Following a 5-day preincubation, the bottles were injected with 150 to 200 μM of *o*-, *m*-, or *p*-cresol from anaerobic filter-sterilized aqueous stock solutions. Experiments were performed in quadruplicate and compared with autoclaved controls. All incubations were at room temperature in the dark.

**Acclimation procedure.** Initial findings on the biodegradation of cresol isomers prompted us to examine *p*-cresol decomposition in greater detail. Aseptically obtained aquifer material (1.2 kg [wet weight]) from either the sulfate-reducing or methanogenic site was placed in separate 1-liter Erlenmeyer flasks with 300 ml of the respective groundwater while inside the anaerobic glove box. Because the groundwater that was added was obtained with the portable well and was sufficiently anaerobic, boiling was not required. However, the groundwater was amended with reductant and resazurin as described earlier. The flasks were sealed with rubber stoppers that were pierced with a severed Balch tube (Bellco Glass, Inc., Vineland, N.J.), as described previously (32). The Balch tube was closed with a 1-cm-thick rubber septum. After sealing, the headspace gas was replaced with a mixture of 80% $N_2$–20% $CO_2$. Aquifer slurries were amended with 200 μM *p*-cresol and incubated at room temperature. When the *p*-cresol was completely metabolized, as determined by high-pressure liquid chromatographic (HPLC) analysis, the aquifer slurries were considered acclimated. Acclimated slurries were able to degrade subsequent additions of *p*-cresol without any detectable lag.

**Characterization of *p*-cresol metabolism.** To characterize *p*-cresol metabolism, aquifer slurries that were acclimated to the degradation of this substrate were subdivided into serum bottles or Balch tubes, while anaerobic conditions were maintained. The rate of *p*-cresol decomposition was then measured in slurries amended with either 2.0 mM $Na_2MoO_4$, 5.0 mM bromoethanesulfonic acid (BESA), or 20 mM $Na_2SO_4$. The effect of hydrogen on *p*-cresol metabolism was tested by replacing the incubation vessel headspace gas with 80% $H_2$–20% $CO_2$ (2 atm). All treatments were performed in duplicate, and autoclaved slurries served as controls.

**Pathway of *p*-cresol metabolism.** The simultaneous adaptation technique described by Stanier (29) was used to help elucidate the pathway of *p*-cresol metabolism in acclimated, sulfate-reducing aquifer slurries. For these experiments, acclimated aquifer solids were combined with a mineral enrichment medium (27) instead of groundwater. The medium was amended with 20 mM $Na_2SO_4$ and 300 to 500 μM of *p*-cresol. After complete biological removal of several additions of *p*-cresol, as determined by HPLC, portions of the enriched aquifer slurry were transferred to sterile serum bottles. Suspected metabolic intermediates were added from sterile anaerobic stock solutions to give a final concentration of 140 to 160 μM for *p*-cresol, *p*-hydroxybenzyl alcohol, *p*-hydroxybenzaldehyde, and *p*-hydroxybenzoate. Phenol and benzoate concentrations were 700 and 550 μM, respectively. The disappearance of substrates was monitored by HPLC.

In addition, we also incubated the suspected intermediates with equimolar concentrations of *p*-cresol in the acclimated aquifer slurries and monitored the rate of *p*-cresol metabolism. Our intent was to test the influence of suspected intermediates on *p*-cresol metabolism and to detect metabolites that might not otherwise have accumulated.

**Effect of oxygen.** The headspace of serum bottles containing acclimated sulfate-reducing aquifer material were adjusted to 100% $O_2$. The rate of *p*-cresol metabolism in these bottles was then compared with others containing a typical headspace gas composition of 80% $N_2$–20% $CO_2$.

**Analytical procedure.** The degradation of substrates and the appearance of metabolites were monitored by HPLC. Liquid samples were taken periodically with a syringe and stored at −10°C until analysis. The thawed samples were centrifuged at 20,000 × *g* for 20 min to remove paticulates prior to HPLC analysis. The separations were performed with an HPLC system (Beckman Instruments, Inc., Berkeley, Calif.) with an organizer (model 340; Beckman) and pumps (dual 110B; Beckman) and a reversed phase column (250 by 4.6 [inner diameter] mm; Econosphere; 5 μm; Alltech Associates, Inc., Deerfield, Ill.). The isocratic mobile phases for the various separations consisted of different ratios of 50 mM acetate buffer (pH 4.5) and acetonitrile. For routine separations, a 90% acetate buffer–10% acetonitrile mixture was used to resolve *p*-hydroxybenzyl alcohol and *p*-hydroxybenzoate, while a 70% acetate buffer–30% acetonitrile mixture was used for the analysis of *p*-hydroxybenzaldehyde and benzoate. Phenol and *p*-cresol were analyzed with a 55% acetate buffer–45% acetonitrile mixture. A flow rate of 1.5 ml/min was used for routine analysis. Compound detection was by UV absorbance by using a variable wavelength detector (Beckman 165; Beckman) operated at 280 nm.

Compound identification was by comparison of HPLC retention times and UV absorption spectra with authentic

chemicals. The UV spectra of authentic and unknown compounds were obtained by first chromatographing the chemicals and then using the rapid scanning capability of the spectrophotometer described above. As a peak passed through the flow cell, the instrument scanned in increments of 5 nm between 200 and 350 nm. Peak resolution was increased for UV scans by reducing the solvent flow rate to 1.0 ml/min and changing the mobile phase mixture to 90 and 85% acetate buffer–10 and 15% acetonitrile for *p*-hydroxybenzoate and *p*-hydroxybenzaldehyde, respectively.

Sulfate was analyzed by anion-exchange HPLC. The HPLC system consisted of a minipump (model 396 LDC/Milton Roy; Laboratory Data Control, Riviera Beach, Fla.), anion-exchange column (250 by 4.6 [inner diameter] mm; Wescan Instruments, Santa Clara, Calif.) conductivity detector (model 213-505; Wescan). The mobile phase was 4.0 mM potassium hydrogen phthalate buffer (pH 4.5) at 2.0 ml/min.

**Chemicals.** All organic chemicals were purchased from Aldrich Chemical Co., Milwaukee, Wis., except for benzoic acid, which was obtained from Sigma Chemical Co., St. Louis, Mo. Chemicals were of the highest purity available and were used without further purification.

## RESULTS

**Biodegradation of cresols in aquifer slurries.** The incubation of *o*-, *m*-, or *p*-cresol in anoxic aquifer slurries resulted in the depletion of the parent substrate after various lengths of time. The lag time before metabolism of the isomers started is reported in Table 1. *o*-Cresol was the most persistent isomer under both MC and sulfate-reducing conditions (SRC), with lag times of over 100 and 90 days in material from the respective sites. Degradation of *m*-cresol began after a shorter period of time, but was generally more persistent under MC (Table 1). The shortest lag times observed were the *p*-cresol. Under SRC, *p*-cresol metabolism started in less than 10 days, whereas 46 days were required before substrate loss was observed in slurries under MC. On subsequent substrate addition, the acclimated rate for *p*-cresol degradation in methanogenic incubations was 18 nmol/h per g (dry weight), while the acclimated rate in sulfate-reducing slurries was 19 times faster (330 nmol/h per g). The short lag times and faster rates of decomposition associated with *p*-cresol caused us to concentrate our efforts on characterizing the metabolism of this substrate.

Table 2 shows that the inhibitor of sulfate-reducing bacteria molybdate (35) reduced the rate of *p*-cresol metabolism by 75% in sulfate-reducing aquifer slurries. However, an inhibitor of methanogenesis, BESA (8), had little influence on the rate of *p*-cresol metabolism in these incubations. A combination of molybdate and BESA reduced the rate of *p*-cresol metabolism to 20%, reflecting the overwhelming influence of the sulfate reduction inhibitor.

In contrast, a very different pattern of inhibition was observed with acclimated methanogenic incubations. In these slurries, BESA reduced the relative rate of *p*-cresol metabolism to 25% of that of the untreated samples, compared with 46% for the molybdate treatments (Table 2). Sulfate addition to methanogenic slurries effectively stimulated the rate of *p*-cresol metabolism by 58%. When the headspace gas of the serum bottles was replaced with a mixture of 80% $H_2$–20% $CO_2$ (2 atm), the rate of *p*-cresol metabolism was reduced by 95% (Table 2).

TABLE 1. Comparison of lag times for the degradation of cresol isomers in anaerobic aquifer slurries

| Substrate | Lag time (days) for: | |
|---|---|---|
| | Sulfate-reducing incubations | Methanogenic incubations |
| *p*-Cresol | <10 | 46 |
| *m*-Cresol | 43 | 46–90 |
| *o*-Cresol | >100 | >90 |

TABLE 2. Effect of various treatments on *p*-cresol degradation in acclimated aquifer slurries

| Treatment | Relative degradation rate (%)[a]: | |
|---|---|---|
| | Sulfate-reducing incubations[b] | Methanogenic incubations[c] |
| None | 100 | 100 |
| 2 mM $MoO_4^{2-}$ | 25 | 46 |
| 5 mM BESA | 97 | 25 |
| BESA–$MoO_4^{2-}$ | 20 | ND[d] |
| 20 mM $SO_4^{2-}$ | 100 | 158 |
| $H_2$[e] | ND | 5 |

[a] Rate of *p*-cresol degradation compared with control with no treatment. See text for actual rates.
[b] Percentage is based on substrate loss over 10 days.
[c] Rate estimate is from slope of best-fit line.
[d] ND, Not determined.
[e] There was 2 atm of headspace pressure of 80% $H_2$–20% $CO_2$.

The relationship between sulfate consumption and *p*-cresol disappearance in acclimated sulfate-reducing aquifer slurries is illustrated in Fig. 1. Results of our previous experiments have shown that it is difficult to measure the small endogenous rate of sulfate removal against the large background by the HPLC technique (Beeman and Suflita, in press). This is also evident from the initial portion of the sulfate curve. Therefore, the sulfate depletion curve in Fig. 1 is uncorrected for the influence of endogenous electron donors. In Fig. 1 it is shown that the rate of depletion for both *p*-cresol and sulfate increases exponentially with time. Comparison of the rates of substrate utilization revealed that about 3.4 mol of sulfate is consumed per mol of *p*-cresol metabolized.

**Pathway of *p*-cresol metabolism under SRC.** In experiments with enriched sulfate-reducing aquifer material, several small peaks were noted on the chromatograms determined by HPLC. The peaks were transient, and one cochromatographed with *p*-hydroxybenzoate, an intermediate detected by other investigators (1, 26; I. D. Bossert and L. Y. Young, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, Q64, p. 268)

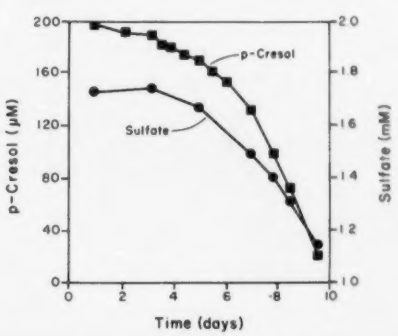


FIG. 1. Sulfate and *p*-cresol depletion in acclimated sulfate-reducing aquifer slurries.

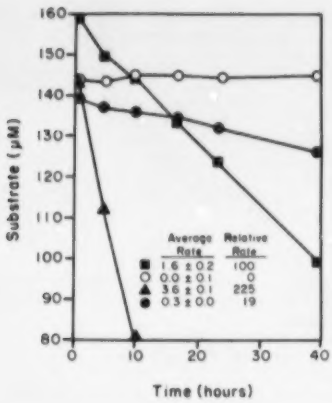


FIG. 2. Simultaneous adaptation of *p*-cresol-acclimated, sulfate-reducing aquifer material to potential metabolic intermediates. Symbols: ■, *p*-cresol; ○, *p*-hydroxybenzyl alcohol; ▲, *p*-hydroxybenzaldehyde; ●, *p*-hydroxybenzoate. The average rate is expressed in micromolar of sample per hour ± standard deviation. The relative rates represent the biodegradation rate relative to that of *p*-cresol.

during *p*-cresol decomposition under methanogenic and nitrate reducing conditions. We therefore performed simultaneous adaptation experiments using substrates that were potential intermediates of *p*-cresol metabolism.

When these compounds were added to acclimated sulfate-reducing slurries, *p*-cresol, *p*-hydroxybenzaldehyde, and *p*-hydroxybenzoate were metabolized without a lag period (Fig. 2). However, *p*-hydroxybenzyl alcohol was not transformed over the 40-h incubation period. It is not known whether any of the postulated intermediates would ordinarily exhibit a substantial lag time in aquifer slurries.

When *p*-hydroxybenzaldehyde was tested as a potential intermediate in the simultaneous adaptation experiments, another peak was detected on the chromatograms determined by HPLC. *p*-Hydroxybenzaldehyde decreased with time and exhibited a first-order decay rate of $0.09 \pm 0.01\ h^{-1}$ over the substrate range tested (Fig. 3). Concomitant with *p*-hydroxybenzaldehyde depletion was the appearance of another peak that cochromatographed with *p*-hydroxybenzoate. The UV spectrum of the metabolite from *p*-

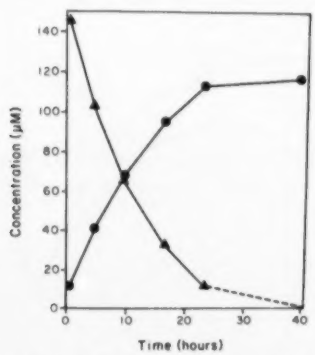


FIG. 3. Dehydrogenation of *p*-hydroxybenzaldehyde (▲) to *p*-hydroxybenzoate (●) in *p*-cresol-acclimated, sulfate-reducing slurries.

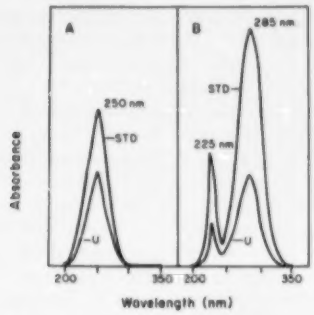


FIG. 4. UV absorption spectra of authentic standards (STD) and metabolites (U). (A) *p*-Hydroxybenzoate. (B) *p*-Hydroxybenzaldehyde.

hydroxybenzaldehyde was compared with the spectrum of authentic *p*-hydroxybenzoate, which was chromatographed under the same conditions. The metabolite and the authentic compound had identical UV spectra, with a single absorbance maximum at 250 nm (Fig. 4A).

When the potential intermediates were incubated with equimolar concentrations of *p*-cresol, it was possible to observe the effects of the intermediates on *p*-cresol degradation and to cause an accumulation of the intermediates that are normally present only in trace quantities. In Table 3 it is shown that *p*-hydroxybenzyl alcohol, *p*-hydroxybenzaldehyde, and *p*-hydroxybenzoate all reduced the relative rate of *p*-cresol degradation to 0, 7, and 48%, respectively. When both *p*-cresol and *p*-hydroxybenzoate were simultaneously incubated in acclimated sulfate-reducing aquifer material, a peak that cochromatographed with authentic *p*-hydroxybenzaldehyde was noted. Similarly, *p*-hydroxybenzyl alcohol was detected when *p*-cresol and *p*-hydroxybenzaldehyde were incubated in the same slurry. In *p*-hydroxybenzoate and *p*-hydroxybenzaldehyde incubations not containing *p*-cresol, the previously detected intermediates did not accumulate. This finding indicates that carbon flow is from *p*-cresol to the newly formed intermediate and not from the added downstream metabolite. The accumulation of *p*-hydroxybenzaldehyde was confirmed by comparison of the UV spectrum of the metabolite with that of the authentic chemical. The two spectra were identical and exhibited maxima at 225 and 285 nm (Fig. 4B). Similar UV scans for the suspected and authentic *p*-hydroxybenzyl alcohol were not performed.

During the biodegradation of *p*-cresol in methanogenic incubations, Senior and Balba (26) reported that *p*-hydroxybenzoate and phenol were formed as metabolic

TABLE 3. Rate of *p*-cresol metabolism in sulfate-reducing aquifer slurries containing an equal molar concentration of the potential intermediate

| Addition | Average rate (μM/h)[a] | Relative rate (%) of degradation |
|---|---|---|
| None | 1.58 ± 0.19 | 100 |
| *p*-Hydroxybenzyl alcohol | 0.01 ± 0.02 | 0 |
| *p*-Hydroxybenzaldehyde | 0.10 ± 0.01 | 7 |
| *p*-Hydroxybenzoate | 0.73 ± 0.01 | 48 |

[a] Average rate was calculated by averaging the rate of two replicate samples and subtracting the rate observed in the autoclaved control. Values are means ± standard deviations.

intermediates. This indicates that a decarboxylation of the *p*-hydroxybenzoate took place. Because anaerobes are known to catalyze both dehydroxylations (33, 38) and decarboxylations (38) of aromatic ring substituents, it is possible that either benzoate or phenol could be formed from *p*-hydroxybenzoate produced during *p*-cresol metabolism. Though these compounds were not detected as intermediates in our experiments, we tested acclimated sulfate-reducing aquifer slurries for their ability to degrade them. Benzoate was metabolized at 6 μM/h without a lag, while phenol was not metabolized even after 40 h of incubation (data not shown).

**Effect of sulfate depletion and oxygen on *p*-cresol metabolism.** The initial stages of the anaerobic decomposition of *p*-cresol that we propose are identical to the aerobic metabolic pathways previously established for this substrate (4, 10–12, 14, 28). Consequently, we questioned the involvement of molecular oxygen as an oxidant of *p*-cresol, even though strict anaerobic techniques were used throughout the study. When the headspace of incubation flasks containing sulfate-reducing aquifer material that was acclimated to *p*-cresol degradation was intentionally replaced with molecular oxygen, the rate of substrate metabolism decreased by 95%.

In other experiments with acclimated aquifer material, repeated additions of *p*-cresol resulted in the depletion of sulfate. When this occurred, *p*-cresol metabolism ceased. The biodegradation of *p*-cresol could not be resumed in these experiments unless the flasks were amended with additional sulfate.

## DISCUSSION

The data presented here demonstrate that several alkyl phenols are degraded under the anaerobic conditions that prevail in polluted aquifers and help explain several field observations. For instance, assuming an equal influence of physicochemical factors in the movement of cresols through the subsurface, the lag times in Table 1 help predict that *o*-cresol would be transported the greatest distance from its point of introduction, while *p*-cresol would migrate the least. Eventually, all cresol isomers should be removed due to microbial degradation. This is the transport pattern and conclusion reached by Goerlitz et al. (7) during their study of alkyl phenol migration in a creosote-contaminated aquifer underlying Pensacola, Fla. Similarly, during an 8-week study of cresol decomposition in anoxic sewage sludge, Boyd et al. (2) observed that *p*-cresol was mineralized to $CH_4$ and $CO_2$ in 3 weeks, while *m*- and *o*-cresol persisted for 7 and more than 8 weeks, respectively.

Our data also suggest that the lag times are shorter and that the degradation rates are faster when cresol isomers are incubated under SRC rather than MC (Tables 1 and 2). In a study of haloaromatic biodegradation with slurries obtained from the same anoxic aquifer, compound metabolism was favored under MC (6a, 30). Such findings illustrate that the nature of the terminal electron acceptor is critical in governing pollutant biodegradation in groundwaters. Perhaps sulfate (or nitrate) could be added to subsurface areas contaminated with alkyl phenols to help stimulate anaerobic decomposition. These electron acceptors are inexpensive and may eventually prove easier to transport in aquifers than oxygen. However, the potential environmental impacts of such materials (e.g., $H_2S$ production) and the nature of organic cocontaminants should be carefully considered in any bioremediation strategy.

Several lines of evidence indicate that *p*-cresol metabolism in sulfate-reducing incubations is coupled to sulfate depletion. First, our characterization of *p*-cresol metabolism showed that an inhibitor of sulfate reduction was also an effective inhibitor of *p*-cresol catabolism (Table 2). In contrast, an inhibitor of methanogenesis did not have a significant influence on *p*-cresol degradation in these incubations. The coupling of *p*-cresol metabolism to sulfate depletion was further illustrated (Fig. 1). As the rate of *p*-cresol degradation increased, so too did the rate of sulfate depletion. A near constant ratio of 3.4 mol of sulfate was consumed per mol of *p*-cresol metabolized (Fig. 1). Assuming *p*-cresol was completely mineralized, the following equation describes the reaction stoichiometry under physiological conditions: $C_7H_8O + 4.25SO_4^{2-} + 3H_2O \rightarrow 7HCO_3^- + 4.25HS^- + 2.75H^+$. The observed rate of sulfate depletion accounts for about 80% of that expected based on this equation. Presumably, some of the *p*-cresol carbon was used to support microbial growth and may help account for the deviation from the theoretical amount reduced.

Lastly, the dependence of *p*-cresol metabolism on sulfate reduction is illustrated in acclimated sediments that deplete their endogenous reserves of sulfate. Further metabolism of *p*-cresol was precluded in these experiments until exogenous sulfate was added to the incubation flasks.

These results lead to questions on the role of sulfate-reducing bacteria in this type of metabolism. This group of microorganisms recently has been found to be more nutritionally diverse than originally believed (36). However, our results do not implicate sulfate reducers as the initiators of *p*-cresol metabolism. During anaerobic metabolism, catalysis is usually not carried out by a single organism, but rather a consortium of bacteria (18). As members of such a consortium, sulfate-reducing bacteria may act as hydrogen-consuming organisms that serve to effectively pull the reaction toward completion. In the case of *p*-cresol, it is unknown if sulfate-reducing bacteria are actually fulfilling this niche. It should be noted that marine sulfate reducers were recently described (36) that can use two of our proposed intermediates, *p*-hydroxybenzoate and benzoate, as suitable electron donors to support sulfate reduction. Our attempts to isolate a *p*-cresol-degrading sulfate reducer however, have thus far proved unsuccessful.

In methanogenic slurries, the blocking of methanogenesis with BESA was more inhibitory to *p*-cresol metabolism than molybdate, indicating the importance of methanogens in the overall flow of *p*-cresol carbon (Table 2). Molybdate also inhibited the transformation of *p*-cresol, even though sulfate was absent from these incubations. Nevertheless, the effect was less than that observed when the same inhibitor was used in sulfate-reducing incubations. Because sulfate-reducing bacteria are known to grow as hydrogen-producing syntrophs coupled with methanogens (3, 19, 22, 24), and because the effect of molybdate on these syntrophs has not been defined, it is possible that molybdate exerts an inhibitory effect on sulfate reducers that live syntrophically in the absence of an electron acceptor. However, it is impossible to attribute the influence of molybdate exclusively to its effect on sulfate reducers.

Sulfate additions increased the rate of *p*-cresol metabolism in methanogenic incubations (Table 2). If sulfate-reducing bacteria were to exist as syntrophs with methanogens, the addition of sulfate could cause a metabolic shift from hydrogen production to consumption via sulfate reduction. Lovley et al. (17) detected a decrease in hydrogen concentration after sulfate addition to methanogenic lake sediment. As

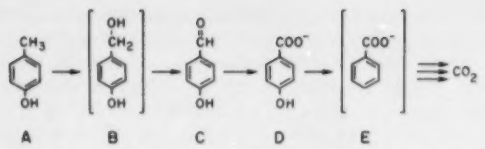


FIG. 5. Proposed pathway of *p*-cresol metabolism under SRC. (A) *p*-Cresol; (B) *p*-hydroxybenzyl alcohol; (C) *p*-hydroxybenzaldehyde; (D) *p*-hydroxybenzoate; (E) benzoate.

noted above, this increased consumption of a metabolic intermediate could conceivably result in a more favorable thermodynamic relationship between substrate and product, effectively pulling the reaction toward completion. However, such an effect need not be strictly associated with a reduction in hydrogen concentration. Other potential metabolites such as acetate, formate, or benzoate could serve in a similar manner.

The detection of HPLC peaks that were chromatographically and spectrophotometrically identical with *p*-hydroxybenzaldehyde and *p*-hydroxybenzoate (Fig. 4) indicates that the methyl substituent of *p*-cresol was initially oxidized under anaerobic sulfate-reducing conditions. A trace peak that cochromatographed with *p*-hydroxybenzyl alcohol was also noted during *p*-cresol metabolism. However, it was not spectrophotometrically compared with authentic material to confirm its identity.

Additional support for the pathway proposed in Fig. 5 is provided by several lines of evidence. First, acclimated sulfate-reducing aquifer slurries utilized *p*-hydroxybenzaldehyde, *p*-hydroxybenzoate, and benzoate without a lag. However, it is important to point out the inherent limitations of the simultaneous adaptation technique (29). A substrate may readily be metabolized, even though it is not an intermediate in a metabolic sequence, if the requisite microbial enzymes are constitutively produced. Conversely, a compound may be a reaction intermediate and yet not be metabolized by whole cell preparations because of permeability barriers or other reasons. Nevertheless, HPLC analysis indicated that several upstream intermediates accumulated when the acclimated slurries were incubated with equal concentrations of *p*-cresol and a downstream metabolite. Lastly, postulated intermediates inhibited *p*-cresol metabolism in a manner consistent with the proposed pathway (Table 3). That is, the metabolites furthest downstream in the *p*-cresol pathway exhibited lesser degrees of inhibition. However, while *p*-hydroxybenzyl alcohol did severely inhibit *p*-cresol degradation, it was not transformed without a lag in the simultaneous adaptation experiments (Fig. 2). Because there are many possible reasons for this result, including the potential toxicity of the intermediate or its inability to penetrate whole cells, we indicated this compound with brackets in the proposed pathway (Fig. 5).

A similar degradation pathway based on anaerobic methyl-group oxidation has also been proposed for *p*-cresol (26) and toluene (D. Grbic-Galic, personal communication) when these substrates are incubated under methanogenic conditions. Bossert and Young (Abstr. Annu. Meet. Am. Soc. Microbiol. 1985) have detected a product resulting from *p*-cresol metabolism under denitrifying conditions which contained a single UV absorption maximum at 255 nm. This result is consistent with our observation of *p*-hydroxybenzoate as an intermediate (Fig. 4B and 5). Interestingly, Hopper (11) demonstrated the incorporation of [$^{18}$O]water into the methyl group of *p*-cresol by a purified methylhydroxylase from an aerobically grown pseudomonad. This enzyme also functioned anaerobically coupled with an artificial electron acceptor (10). Under these conditions, the enzyme transformed *p*-cresol to the corresponding aldehyde. The *p*-hydroxybenzyl alcohol was only released from the enzyme when the electron acceptor was left out of the reaction mixture (11).

The metabolic pathway shown in Fig. 5 is somewhat different from that suggested by several investigators (26, 39) in that we do not propose phenol as an intermediate of *p*-cresol metabolism. Aquifer slurries acclimated to *p*-cresol degradation were able to transform benzoate, but not phenol, without a lag. Therefore, we propose that *p*-hydroxybenzoate is dehydroxylated rather than decarboxylated under SRC. Healy and Young (9) observed that mixed methanogenic cultures acclimated to the degradation of *p*-hydroxybenzoate cross-acclimated to both phenol and benzoate. Taylor et al. (34) demonstrated the dehydroxylation of *p*-hydroxybenzoate to benzoate with a facultative nitrate-reducing bacterium. However, because benzoate was not actually detected as a free intermediate in our study, it too is listed as a metabolite in brackets in Fig. 5.

The proposed metabolites resulting from *p*-cresol decomposition under SRC are similar to those that would result from an aerobic methyl oxidation (4, 10, 14–16, 20, 21, 28, 37). However, molecular oxygen is not involved as a reactant in the proposed pathway because its intentional addition to the incubation severely inhibited *p*-cresol metabolism. Incubation vessels also contained resazurin as a sensitive redox indicator, and no trace of pink coloration was ever detected visually. Lastly, it is difficult to explain the cessation of *p*-cresol metabolism in sulfate-depleted aquifer slurries if $O_2$ was the participating oxidant; it is also difficult to explain the continuation of degradation only when sulfate was reamended to these flasks. Experiments with [$^{18}$O]water are planned to help substantiate this contention, and $^{14}$C-labeled *p*-cresol will be used to further probe the pathway proposed in Fig. 5. It is most likely that *p*-cresol is oxidized under anaerobic conditions by a methylhydroxylase.

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## ADDENDUM IN PROOF

Recently, two isolates have been described which can metabolize *p*-cresol under anaerobic conditions. I. D. Bossert and L. Y. Young (Appl. Environ. Microbiol. **52**:1117–1122, 1986) have described a denitrifying bacterium that oxidizes the methyl group of *p*-cresol to form *p*-hydroxybenzoic acid. F. Bak and F. Widdel (Arch. Microbiol. **146**:177–180, 1986) have isolated a marine bacterium able to mineralize *p*-cresol by using sulfate as the terminal electron acceptor.

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